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ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF ENVIRONMENTAL BACTERIAL ISOLATES WITH SCREENING FOR ANTAGONISM AGAINST THREE BACTERIAL TARGETS

by
Robert Stote
Jennifer M. Rego
and
Romy Kirby

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October 2012 – September 2013

U.S. Army Natick Soldier Research, Development and Engineering Center
Natick, Massachusetts 01760-5020

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Current antimicrobial treatments exhibit a broad range of killing power, promoting the increase of multi-drug resistant organisms. This has led to the urgent need to develop targeted antimicrobials as an alternative to today's treatments. This report summarizes work conducted to identify microorganisms that exhibit narrow-spectrum activity through the secretion of antimicrobials, termed bacteriocins, from a pool of environmental isolates collected at Fort Devens. The environmental isolates were characterized and found to be comprised mostly of microorganisms from the genus *Bacillus* and *Staphylococcus*. The environmental isolates were screened for bacteriocin-induced activity against three target strains of interest to the DoD: *Bacillus anthracis* Sterne, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The percentage of environmental isolates that demonstrated activity against *Bacillus anthracis* Sterne was 15% (9 of 62 isolates screened), while 2% of the isolates (2 of 114 isolates screened) exhibited activity against *Staphylococcus aureus*. No isolates were active against *Pseudomonas aeruginosa*. The active isolates were screened further against additional targets to confirm their narrow-spectrum activity. This work successfully identified environmental microorganisms that exhibit bacteriocin-driven activity to produce narrow-spectrum antimicrobials that target DoD relevant microorganisms.

15. SUBJECT TERMS

SPORES	ISOLATION	BACILLUS ANTHRACIS	ENVIRONMENTAL ISOLATES
CODING	SELECTION	RESISTANCE(BIOLOGY)	TARGETED ANTIMICROBIALS
ISOLATES	BACTERIOCINS	ANTIMICROBIAL AGENTS	PSEUDOMONAS AERUGINOSA
TARGETS	IDENTIFICATION	MULTI-DRUG RESISTANCE	NARROW SPECTRUM ANTIBIOTICS
ASSAYING	NOMENCLATURE	STAPHYLOCOCCUS AUREUS	
BACTERIA	MICROORGANISMS	MULTI-DRUG RESISTANT ORGANISMS	
BIOASSAY	CHARACTERIZATION	NARROW-SPECTRUM ANTIMICROBIALS	

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Table of Contents

List of Tables

Preface

This report documents work by the Biological Sciences and Technology Team (BSTT) of the Warfighter Directorate at the Natick Soldier Research, Development and Engineering Center (NSRDEC) during the period from October 2012 to September 2013. This work involved initial investigations of narrow-spectrum antimicrobial agents, termed bacteriocins, to facilitate their future incorporation into materials and textiles. The objective of this work was to isolate a pool of bacteria from the environment and subsequently evaluate the bacteriocin-induced activity against three target microorganisms of interest to the military: *Bacillus anthracis* Sterne, *Pseudomonas aeruginosa,* and *Staphylococcus aureus*. Of the over 100 environmental isolates collected, 11 environmental isolates were identified as possessing activity against *Bacillus anthracis* Sterne and *Staphylococcus aureus* while no isolates were identified as being active against *Pseudomonas aeruginosa*.

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ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF ENVIRONMENTAL BACTERIAL ISOLATES WITH SCREENING FOR ANTAGONISM AGAINST THREE BACTERIAL TARGETS

1 Introduction

The Biological Sciences and Technology Team (BSTT), Warfighter Directorate, of the Natick Soldier Research, Development and Engineering Center (NSRDEC) is investigating the use of narrow-spectrum antimicrobials in non-traditional textiles to protect the Warfighter from pathogenic bacteria. This investigation has several parts, one of which is to identify environmental bacterial isolates that produce bacteriocins, a class of narrow-spectrum antimicrobials, against specific targets. The objective of this technical report, is to describe the generation and characterization of a bacterial pool of environmental isolates and the subsequent screen of the pool for bacteriocin-driven antimicrobial activity against military relevant pathogenic target strains of bacteria. The work was performed from October 2012 to September 2013.

As background, drug resistant strains of bacteria are on the rise, while the number of drugs being developed against them is declining (Cooper and Shlae, 2011). This creates a situation where infections that are currently treatable may not be in the very near future. Additionally, many of these drug resistant strains demonstrate resistance to the antimicrobials used in textiles, polymer surfaces, wipes, and ointments, making the agent ineffective (Tattawasart *et al*. 1999, Thomas *et al*. 2000, McDonnell and Russell, 1999, Silver, 2003). Though a number of hypotheses have been offered to explain this increase in resistance, one of the leading causes has been identified as the use of broad-spectrum antimicrobials that unintentionally promote the selection of bacterial strains resistant to the antimicrobial (Levy and Marshall, 2004). When broad-spectrum antimicrobials are employed, not all of the bacteria are killed. Those that have developed a resistance to the antimicrobial will survive. Since the more benign microorganisms that contribute to keeping drug-resistant pathogenic strains under control are killed off, the resistant strains are allowed to grow unfettered (Levy and Marshall, 2004, Gulberg *et al*. 2011). After a few exposures to a broad-spectrum antimicrobial, the collection of bacteria becomes primarily composed of resistant strains, making the broad-spectrum antimicrobial ineffective. When this occurs, the current strategy is to replace an ineffective antimicrobial agent with another broad-spectrum antimicrobial agent. This provides an opportunity for the bacterial pool to be further screened for those bacteria that are now resistant to the new antimicrobial agent as well as the previous antimicrobial, leading to the development of multi-drug resistant bacteria, or “super bugs”. As concern about the emergence of multi-drug resistant bacteria increases, new strategies are being investigated to inhibit the development of resistant strains. One promising new strategy is to use narrow-spectrum antimicrobials, such as bacteriocins, as alternatives to broad-spectrum agents.

Bacteriocins are narrow-spectrum bacteria toxins secreted by bacteria to kill other closely related bacteria that are competing for the same resources. Many bacteriocins have very specific

activity spectrums, as they only target one or two species. This selectivity offers a paradigm shift from the typical application of conventional antimicrobials where instead of broadly killing everything, specific pathogens could be targeted, leaving beneficial bacteria unaffected and capable of thriving (Abt and Pamer, 2014). Generally considered a green alternative to the currently used antimicrobials, few bacteriocins have demonstrated adverse effects on eukaryote cells (Cotter *et. al.*, Cox *et. al*., Galvz *et al.*). These and other characteristics make bacteriocins an appealing option to supersede broad-spectrum antimicrobials.

Selecting bacteriocins for Army-specific needs requires the screening of large pools of environmental isolates to obtain two pieces of information: (1) is the activity against the desired target observed, and (2) how broad is the activity toward other organisms? From this information, environmental isolates that display bacteriocin-generating activity against pathogenic bacteria can be identified for further characterization. This would lead to the eventual purification and application of the bacteriocin itself. In this reported study, a pool of environmental isolates was generated, identified to the genus level, and screened for bacteriocin activity. Identification of the environmental isolates was achieved via phenotypical analysis as well as analysis by the Omnilog system. To evaluate activity, three military relevant microorganisms were selected as targets: *Bacillus anthracis* Sterne, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The environmental isolates were screened against these targets for bacteriocin-like activity.

2 Materials and Methods

2.1 Isolation of Environmental Pool

A pool of bacterial isolates was collected from several locations at Fort Devens (Ayer, MA). 12 sites of interest were wiped with sterile cotton swabs, including air conditioning (AC) ducts, a door handle, a cot, a cutting board, a kitchen sink, and the handle of a washing machine. The swiped cotton swabs were then used to inoculate nutrient agar plates. The plates were incubated at 37 ^{o}C for 24 h. Individual colonies were picked from each plate using a sterile loop, and were streaked onto a new nutrient agar plate to obtain a pure isolate. The initial plates were then incubated again for another 24 h to allow any slow-growing colonies to develop. Newly developed colonies were picked and streaked onto a new nutrient agar plate to obtain a pure isolate.

2.2 Colony characterization

Identification of environmental isolates followed the flowchart from "Bergey's Manual of Determinative Bacteriology" (Holt *et al*. 1994), which utilizes physical and biochemical analysis to identify the genus, and sometimes species, of unknowns. These tests are well-established methods utilized to identify unknown bacteria samples and have been well-documented in previous literature. These physical and biochemical tests include: (1) the Gram stain test, which is used to determine if the organism is Gram-negative or Gram-positive; (2) the acid-fast test, which identifies *Mycobacterium*; (3) the oxidase test, which identifies organisms that produce the enzyme cytochrome oxidase; (4) the catalase test, which differentiates *Staphylococci* (a catalase positive organism) from *Streptococci* (a catalase negative organism); (5) the starch hydrolysis test, which evaluates the organism's ability to synthesize and secrete enzymes to break down starch into smaller subunits to be used by the organism; (6) the mannitol and glucose fermentation test, which investigates how an organism metabolizes sugars; (7) the Voges-Proskauer test, which tests the organism's ability to produce non-acidic end products from the metabolism of glucose and is helpful in differentiating the *Enterobacteriaceae*; (8) the citrate test, which studies the organism's ability to apply citrate as a carbon source and is also helpful in differentiating the *Enterobacteriaceae*; (9) the nitrate reductase test, which investigates bacteria's ability or inability to reduce nitrate to nitrite; (10) the novobiocin sensitivity test, which evaluates an organism's susceptibility to novobiocin, an antibiotic that obstructs DNA replication and is helpful in differentiating among Gram-positive cocci; (11) the diffusible pigment test using king agar, which differentiates among species of *Pseudomonas* by testing the secretion of pigments by the organism. Additionally, the bacteria isolates were viewed under a standard microscope to characterize their shape. The Omnilog Identification system, which uses a colorimetric assay based upon the consumption of different carbon sources, was employed to further characterize the pool of environmental microorganisms.

Materials for physical and biochemical analysis were sourced from several different companies:

- Gram stain from ENG Scientific, Clifton, NJ
- Acid-fast from Electron Microscopy Sciences, Hatfield, PA
- Oxidase from Becton, Dickinson and Company (BD), Sparks, MD
- Starch hydrolysis from ENG Scientific, Clifton, NJ
- Mannitol and glucose fermentation from Sigma Aldrich, St Louis, MO

- Voges-Proskar test kit from BD, Sparks, MD
- Citrate from Sigma Aldrich, St Louis, MO
- Novobiocin sensitivity from Remel, Lenexa, KS
- Diffusible pigment test using king agar from Sigma-Aldrich, St Louis, MO
- Gen III Omnilog system and any associated supplies from Biolog, Inc., Hayward, CA

2.3 Screening Isolates for Activity of Bacteriocins Against Targets

Selected isolates were screened for bacteriocin activity against three targets: *Bacillus anthracis* Sterne (surrogate for *Bacillus anthracis*), *Staphylococcus aureus* (ATCC 27217), or *Pseudomonas aeruginosa* (ATCC 15692) using soft agar overlays. A culture containing the target microorganism and a culture of the environmental isolate to be tested were inoculated into nutrient broth and incubated at 37 ^{o}C until an Optical Density (OD) of 1 (~10^8 cfu/mL) was achieved. Soft agar for overlay experiments was prepared with 7% agar in nutrient broth. Overlays were prepared by adding 60 µL of the target organism with 1.35 µL of a 100 mg/mL stock mitomycin C solution (from Amresco Inc., Solon, OH) to a 7 mL aliquot of soft agar. Since some bacteriocin are produced as a threat response, mitomycin C was added to induce their production. The isolate was tested by dropping 6 µL of culture onto each plate in duplicate. The plates were incubated at 37 ^{o}C overnight. Positive activity was determined by the presence of a zone of clearing.

3 Results and Discussion

3.1 Isolation and Identification of Environmental Bacterial Isolates

Table 1 lists the surfaces swabbed at Fort Devens, their specified sample name, and the total number of unique bacteria colonies isolated. Four of the surfaces investigated did not yield any growth of bacteria colonies. These surfaces were the tent canvas (FD1 and FD4), a door handle (FD5) and a scarcely used AC duct (FD9). The lack of bacteria colonies isolated from this door handle and AC duct may be due to the infrequent use of both surfaces, which limited the interactions necessary for bacteria transfer from the environment to the surface. The absence of bacteria colonies isolated from the tent fabric may be due in part to the fact that the shelter was assembled only a month before the samples were collected. This short period of time may not have allowed sufficient exposure of the surface to the elements and people for microorganisms to be detected. Additionally, tent materials are treated with a water repellant and an antifungal treatment, which may inhibit the adhesion and/or growth of bacteria. Because the kitchen was recently set up and had not yet been used, it was expected that not many bacterial isolates would be present, as the results depict. It is anticipated that re-visiting the kitchen to swab surfaces after the kitchen has been used for meal preparation would yield different results, including the isolation of more microorganisms.

Table 1. Summary of Surfaces Probed for Environmental Bacterial Isolates

Sample Name	Surface	Number of Colonies Isolated
FD1	tent canvas	0
FD2	tent 6 cot metal handle	1
FD3	tent 6 AC duct return	2
FD4	tent canvas	0
FD5	door handle	0
FD6	tent 8 AC duct return	39
FD7	rigid shelter door handle	31
FD8	rigid shelter air return	24
FD9	AC duct return (barely used)	0
FD10	laundry 47 washer handle	9
FD11	kitchen sink and handles	7
FD12	kitchen cutting board	1

Following the isolation of environmental bacterial isolates, they were characterized. The Appendix depicts the results of the physical and biochemical tests carried out following Bergey's Manual of Determinative Bacteriology to identify each microorganism. Using this methodology, all but nine of the isolates were identified to their genus. A total of 114 unique isolates were identified.

To confirm the genus identification results of the isolates as determined by the physical and biochemical tests, and to also assist with identifying the species of the isolated organisms, the Omnilog Identification system (Omnilog) was used. The Omnilog was able to positively identify the genus of 54% of the isolates (61 of 114 isolates), with a few to the species level (Appendix). The Omnilog was unable to identify any of the nine unknowns. The failure of the Omnilog system to identify the genus or species for a greater number of environmental isolates may be due to the limitations of the Omnilog database. The Omnilog database consists of information for primarily clinical microorganisms and not environmental microorganisms, like the bacterial isolates used in this study. Though not done in this work, an alternative method, such as genomic sequencing, would be more effective to confirm the genus identification results obtained from biochemical and physical analysis. This should be considered for future efforts.

Table 2 summarizes the results of genus identification through physical and biochemical analysis as well as Omnilog studies for each of the 114 unique environmental isolates collected. Excluding the unidentified organisms, seven distinctive genera were identified. Bacteria from the genus *Bacillus* and *Staphylococcus* were present in the greatest amount at 47% and 25% respectively. Conversely, only one isolate was identified from the genus *Aquaspirillum* and *Brevibacillus*. The greatest number of isolates were obtained from two of the AC ducts (57%) and the door handle (27%). Of the total number of isolates collected from the two AC ducts, 60% were identified as the genus *Bacillus*. These results are to be expected as AC ducts contain a significant amount of debris on their interior surfaces and the spore forming bacteria *Bacillus* can easily become embedded in the interior of the ducts. Of the total number of isolates collected from the door handle, 84% were identified as the genus *Staphylococcus*. As *Staphylococcus* make up a significant portion of the skin microbiota, it is reasonable to infer that the large quantity collected from the door handle is due to the frequent interaction of people's hands with the door handle.

Table 2. Summary of Genus Identification of Environmental Bacterial Isolates in Relation to Sample Collection Location

	Frequency								
	All Surfaces	Laundry	Kitchen sink	Cutting board	Tent 6 cot	Tent 6 AC	Tent 8 AC	Rigid shelter door handle	Rigid shelter AC
Organism Genus									
Aquaspirillum	1								1
Bacillus	53	4	7	1	1	2	23	1	14
Brevibacillus	1								1
Cupriavidus	11						11		
Micrococcus	7							2	5
Pseudomonas	3	1					2		
Staphylococcus	29	2						26	1
Unknown	9	2					3	2	2
Total	**114**	**9**	**7**	**1**	**1**	**2**	**39**	**31**	**24**

3.2 Characterization of Activity of Environmental Bacterial Isolates Against the Target Bacteria

Table 3 shows a summary of the number of isolates that exhibited activity against the target strains evaluated. Since bacteriocins typically only demonstrate activity against close competitors, initial screens of bacteriocin activity against the target microorganisms focused on the same genus. *Bacillus* isolates and the nine unknowns were screened for activity against a *Bacillus anthracis* Sterne target; *Staphylococcu*s isolates and the nine unknowns were screened for activity against a *Staphylococcus aureus* target (ATCC 27217), and *Pseudomona*s isolates and the nine unknowns were screened for activity against a *Pseudomonas aeruginosa* target (ATCC 15692). None of the unknowns showed activity against any of the three targets. Of the 53 *Bacillus* isolates, 15% or 9 of the 62 isolates tested showed activity against the *Bacillus anthracis* Sterne target. Only one *Staphylococcus* isolate showed activity against the *Staphylococcus aureus* target. None of the *Pseudomonas* isolates exhibited activity against the *Pseudomonas aeruginosa* target. Secondary activity screens were performed to determine if any of the isolates produced bacteriocins against bacteria from the other target genera. All the isolates were screened against the *Staphylococcus aureus* and *Pseudomonas aeruginosa* targets. Only one of the isolates, a *Bacillus*, showed activity against the *Staphylococcus aureus* target, while no isolates showed activity against the *Pseudomonas aeruginosa* target. Thus, only 2% or 2 out of the 114 isolates tested were found to be active against the *Staphylococcus aureus* target. Table 4 summarizes in greater detail the results of the isolates that were found to be active against the tested target strains.

Table 3. **Summary of Activity of Isolates Against Target Microorganisms**

Target Organism	Total isolates screened	Number of isolates positive for activity
Bacillus anthracis Sterne	62	9
Staphylococcus aureus	114	2
Pseudomonas aeruginosa	114	0

Table 4. Detailed Summary of Active Isolates

#	Name	Organism Identity	Target Active Against
62	FD6 b	*Bacillus subtilis*	BA
65	FD6 e	*Bacillus*	BA
142	FD8 o	*Bacillus*	BA
148	FD8 u	*Bacillus thuringensis*	BA
151	FD8 x	*Bacillus licheniformis*	BA
157	FD10 f	*Bacillus thuringensis*	BA
162	FD11 b	*Bacillus*	BA
164	FD11 d	*Bacillus*	BA
165	FD11 e	*Bacillus*	BA
60b	FD3 b	*Bacillus*	SA
105	FD7 g	*Staph*	SA

BA: *Bacillus anthracis* Sterne, SA: *Staphlocooccus aureus* (ATCC 27217)

4 Conclusions

A low percentage of environmental isolates displayed bacteriocin activity against the tested targets, but this finding was expected. When interpreting the bacteriocin activity results, one should keep in mind that the isolates were initially screened against a single target strain for each genera. Though most bacteria produce bacteriocins, generally they only produce bacteriocins against bacteria strains that exist as competitors in their environment, which contributes to the narrow activity spectrum of bacteriocins. Thus, a low number of isolates were anticipated to be active against the target bacteria strains. Within that context, the *Bacillus sp.* bacteria strains isolated from environmental sources were expected to yield activity against the *Bacillus anthracis* Sterne target since *Bacillus anthracis* is a soil-borne bacteria. Nine isolates of *Bacillus* did in fact produce bacteriocins against the *Bacillus anthracis* Sterne target. Additionally, characterizing a single strain of *Staphylococcus* with activity against the target strain of *Staphylococcus* was not surprising given that most of the *Staphylococcus* isolates were from the same door handle and may be the same species/strain. What was more interesting was the unique finding of one *Bacillus* isolate that was active against the *Staphylococcus* target, yet inactive against the *Bacillus anthracis* Sterne target. The results from the spectrum screen expand this uniqueness in that the isolate did not show activity toward any of the *Bacillus* strains tested. Further study of this isolate is warranted to understand why it is active against the *Staphylococcus* target and not its *Bacillus* competitors.

The narrow-spectrum activity of the 11 environmental isolates found to demonstrate bacteriocin activity against the target strains was further confirmed with the assistance of a collaborator at the University of Massachusetts, Amherst. The 11 active isolates identified in the initial screening (Table 4) were tested for activity against additional targets of different genera and strains to characterize the extent of activity displayed by the isolates. The results of these additional activity screens showed that the 11 isolates exhibited no activity against unrelated genus strains and only minimal activity against varying strains of the same genus (results not shown). These findings provide additional support to infer that the bacteriocin activity of the environmental isolates is specific to a narrow-spectrum range of activity. Thus, the nine isolates identified as active against *Bacillus anthracis* Sterne and the two isolates that demonstrated activity against *Staphylococcus* resulting from this study are excellent candidates for development as narrow-spectrum antimicrobials to specifically target pathogens of interest to the DoD.

The low number of environmental isolates found to be active against the target strains evaluated illustrates the narrow-spectrum activity of bacteriocins. Moreover, screening of the active isolates against additional organisms revealed minimal activity, which further validates the specific bacteriocin-driven activity of the environmental isolates. The active environmental bacteria isolates provide a valuable resource for narrow-spectrum antimicrobial agents. Future studies will build upon this work by isolating, purifying, and characterizing bacteriocins produced from these active environmental bacteria isolates.

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Appendix:
Results of Physical and Biochemical Analysis to Determine Identity of Environmental Isolates

#	Name	Organism Identity	Gram	Shape	Spore	Swollen	Catalase	Starch Hyd	Oxidase	Glucose Ferm	VP	Novo-biocin	Citrate	Growth in 6.5% Na	Nitrate Reduction
59	FD2 a	*Bacillus*	+	Rod	Yes	+	+	+		X	-				
60	FD3 a	*Bacillus*	+	Rod	Yes	+	+	+			-			-	
61	FD6 a	*Bacillus*	Variable	Rod	Yes	+	+	+	+	-	X				
62	FD6 b	*Bacillus*	+	Rod	Yes		+	+							
63	FD6 c	*Bacillus*	+	Rod	Yes	-	+	+			-		X		
64	FD6 d	*Cupriavidus*	-	Rod					+	-					
65	FD6 e	*Bacillus*	+	Rod	Yes		+	+							
66	FD6 f	*Bacillus*	+	Rod	Yes	-	+	-							
67	FD6 g	*Bacillus*	+	Rod	Yes	-	+	-							
68	FD6 h	*Cupriavidus*	-	Rod					+	-					
69	FD6 i	*Bacillus*	+	Rod	Yes	+	+	-							
70	FD6 j	*Cupriavidus*	-	Rod					+	-					+
71	FD6 k	*Bacillus*	+	Rod	Yes			+							+ beforeZn
72	FD6 l	*Bacillus*	+	Rod	Yes	+	+	-							
73	FD6 m	*Bacillus*	+	Rod	Yes	-	+	+			-		X		
74	FD6 n	*Cupriavidus*	-	Rod					+	-					
75	FD6 o	*Pseudomonas*	-	Rod	Maybe				+	-					-
76	FD6 p	*Cupriavidus*	-	Rod					+	-					X
77	FD6 q	*Cupriavidus*	Variable	Rod	Yes		+	-							
78	FD6 r	*Cupriavidus*	-	Rod			+		+	-					
79	FD6 s	*Bacillus*	+	Rod	Yes	+			+	-					

80	FD6 t1	*Bacilus*	+	Rod	Yes	-	+	-							
81	FD6 t2	*Bacillus*	Variable	Rod	Yes	Yes	+	+			-			-	
82	FD6 u	*Bacillus*	+	Rod	Yes	Yes	+	+			-				
83	FD6 v	*Bacillus*	+	Rod	Yes	-	+	-						-	
84	FD6 w	*Bacillus*	+	Rod	Yes	-	+	-							-
85	FD6 x1	*Bacillus*	+	Rod	Yes		+	+			+		X		
86	FD6 x1	*Bacillus*	Variable	Rod	Yes	-	-	+			-				+ before Zn
87	FD6 y	*Bacillus*	Variable	Rod	Yes		+	+			-				
88	FD6 z	*Cupriavidus*	-	Rod											
89	FD6 aa	*Cupriavidus*	-	Rod	Maybe		+		+	-					
90	FD6 bb	*Bacillus*	+	Rod	Yes		+	-							+
91	FD6 cc	*Bacillus*	Variable	Rod	Yes	-	+	-	+	-				-	
92	FD6 dd		Variable	rod	Yes	Yes	+					X			
93	FD6 ee	*Bacillus*	+	Rod	Yes		+	+						+	
94	FD6 ff	*Bacillus*	Variable	Rod	No	X	+	-							+ before Zn; frothy
95	FD6 gg		-	Rod					+	-					
96	FD6 hh	*Bacillus*	+	Rod	Yes	X	+	-						-	
97	FD6 ii	*Cupriavidus*	-	Rod			+		+	-					
98	FD6 jj	*Cupriavidus*	-	Rod					+	-					
99	FD7 a	*Staph*	+	Cocci			+								
100	FD7 b	*Staph*	+	Cocci			+					X			+ after Zn
101	FD7 c	*Staph*	+	Cocci			+					+			
102	FD7 d	*Staph*	+	Cocci			+			-		+			

103	FD7 e	*Staph*	+	Cocci			+					+			
104	FD7 f	*Staph*	+	Cocci			+					+			
105	FD7 g	*Staph*	+	Cocci			+					X			
106	FD7 h	*Staph*	+	Cocci			+					+			
107	FD7 i	*Staph*	+	Cocci			+					+			
108	FD7 j	*Staph*	+	Cocci			+					+			
109	FD7 k	*Staph*	+	Cocci			+								
110	FD7 l	*Staph*	+	Cocci			+					+			
111	FD7 m	*Staph*	+	Cocci			+					X			
112	FD7 n	*Staph*	+	Cocci			+								
113	FD7 o	*Staph*	+	Cocci			+					+			+ w/ gas
114	FD7 p	*Micrococcus*	+	Cocci			+			-				-	
115	FD7 q	*Staph*	+	Cocci			+					+			+ after Zn; frothy
116	FD7 r	*Staph*	+	Cocci			+					+			
117	FD7 s	*Staph*	+	Cocci			+					+			
118	FD7 t1	*Staph*	+	Cocci			+					+			
119	FD7 u	*Staph*	+	Cocci			+			-					
120	FD7 v	*Staph*	+	Cocci			+					+			
121	FD7 w	*Staph*	+	Cocci			+					+			
122	FD7 x	*Staph*	+	Cocci			+					+			
123	FD7 y	*Staph*	+	Cocci			+					+			
124	FD7 z	*Staph*	+	Cocci			+					+			
125	FD7 aa	*Staph*	+	Cocci			+					+			
126	FD7 bb						+								
127	FD7 cc	*Micrococcus*	+	Cocci			+			-					
128	FD8 a	*Bacillus*	+	Rod	Yes		+	+							
129	FD8 b	*Bacillus*	Variable	Rod	Yes	+	+	-							

130	FD8 c	*Micrococcus*	+	Cocci			+			-				+	
131	FD8 d	*Bacillus*	+	Cocci			+					-			
132	FD8 e	*Micrococcus*	+	Cocci			+			+					
133	FD8 f	*Aquaspirillum*	-	Rod					+	-					
134	FD8 g	*Bacillus*	Variable	Rod	Yes	+	+	+	+	+	-	+			
135	FD8 h	*Bacillus*	+	Rod	Yes		+	+			X				
136	FD8 i	*Bacillus*	+	Rod	Yes	+	+	-						-	
137	FD8 j	*Bacillus*	Variable	Rod	Yes	Maybe	+	+			-				-
138	FD8 k														
139	FD8 l	*Micrococcus*	+	Cocci			+			-				+	
140	FD8 m	*Bacillus*	+	Rod	Yes	X	+	-							
141	FD8 n	*Micrococcus*	Variable	Cocci			+			-					
142	FD8 o	*Bacillus*	+	Rod	Yes				+	-				-	
143	FD8 p	*Bacillus*	+	Rod	Yes	Yes	+	Maybe	+	-					+ before Zn
144	FD8 q	*Bacillus*	+	Rod	Yes		+	-							
145	FD8 r	*Brevibacillus*	Variable	Rod					+	-					
146	FD8 s														
147	FD8 t	*Micrococcus*	+	Cocci			+			-					
148	FD8 u	*Bacillus*	+	Rod	Yes		+	+							
149	FD8 v	*Bacillus*	+	Rod	Yes	+	+	+	+	+	-			-	
150	FD8 w	*Staph*	+	Cocci			+					+			
151	FD8 x	*Bacillus*	+	Rod	Yes		+	Lost plate							
152	FD10 a	*Bacillus*	+	Rod	Yes	-	+	+			-				X
153	FD10 b	*Pseudomonas*	-	Rod			+		+	-					-

154	FD10 c	*Bacillus*	+	Rod	Yes		+	+							
155	FD10 d	*Bacillus*	+	Rod					+	-					
156	FD10 e	*Staph*	+	Cocci			+					+			
157	FD10 f	*Bacillus*	+	Rod	Yes		+	+							
158	FD10 g		-	Rod					+	+					
159	FD10 h	*Staph*	+	Cocci			+					+ ?			
160	FD10 i	*Nocardia?*	-	Mixed	Yes	Not swollen	+		Positive						
161	FD11 a	*Bacillus*	+	Rod	Yes	X	+	+			-				+ after Zn
162	FD11 b	*Bacillus*	+	Rod	Yes	-	+	-							
163	FD11 c	*Bacillus*	+	Rod	Yes	+	-	+			-				
164	FD11 d	*Bacillus*	+	Rod	Yes	-	+	+			-				
165	FD11 e	*Bacillus*	+	Rod	Yes	Maybe	+	+			-				
166	FD11 f	*Bacillus*	Variable	Rod	Yes	-		+	+	+	-				
167	FD11 g	*Bacillus*	+	Rod	Yes		+	-							
168	FD12 a	*Bacillus*	+	Rod	Yes	+	+	+			-				X
116 a	FD7ra	*Bacillus*	X	X	Yes	+	+								
118 b	FD7 t2		+	Cocci			+								
60b	FD 3b	*Bacillus*	+	Rod	Yes	Yes	+	-			+				
71b	FD6K b		+	Cocci or rod	No	?	+								